(19)日本国特許庁（ＪＰ）　(12) 公開特許公報（A）　(11)特許出願公開番号

特開平6－292592

(43)公開日　平成６年(1994)10月21日

(51)Int.Cl.[5]	識別記号	庁内整理番号	ＦＩ	技術表示箇所
Ｃ１２Ｐ　21/00		Ａ　8214－4Ｂ		
21/08		8214－4Ｂ		
//（Ｃ１２Ｐ　21/00				
Ｃ１２Ｒ　1:91）				
（Ｃ１２Ｐ　21/08				

審査請求　未請求　発明の数１　ＦＤ　（全７頁）　最終頁に続く

(21)出願番号　特願平6－31019

(22)出願日　平成６年(1994)２月２日

(31)優先権主張番号　特願平5－44587

(32)優先日　平５(1993)２月９日

(33)優先権主張国　日本（ＪＰ）

(71)出願人　000006699
雪印乳業株式会社
北海道札幌市東区苗穂町６丁目１番１号

(72)発明者　立花　宏文
福岡県福岡市南区塩原２－７－28

(72)発明者　村上　浩紀
福岡県福岡市東区名島４－16－16

(72)発明者　新本　洋士
埼玉県川越市旭町２－13－２－416

(72)発明者　堂迫　俊一
埼玉県浦和市北浦和５－15－39－616

(74)代理人　弁理士　藤野　清也

(54)【発明の名称】　糖蛋白質の生産方法

(57)【要約】

【構成】　動物細胞を培地中で培養して糖蛋白質を生産するに当り、培地中の糖組成あるいは糖濃度を変更することによって生産される糖蛋白質の糖鎖の種類あるいは分子量を改変する方法。

【効果】　培地中の糖組成、糖濃度の変更によって糖蛋白質の糖鎖を改変することができ、種々の性質の異なる糖蛋白質を簡単な手法で任意に得ることができる。

－　－

1

【特許請求の範囲】

【請求項1】　培地中で動物細胞を培養し糖蛋白質を生産するに当り、培地中の糖組成および／または糖濃度を変えることにより、蛋白質に結合する糖鎖の種類あるいは分子量を改変することを特徴とする糖蛋白質の生産方法。

【発明の詳細な説明】

【0001】

【産業上の利用分野】本発明は、培地中で動物細胞を培養して糖蛋白質を生産する際に、細胞の培養条件、特に培地条件を制御することにより、蛋白質に結合する糖鎖の種類あるいは分子量を改変し、特性の変化した糖蛋白質を生産する方法に関する。

【0002】

【従来の技術】リンホカイン、サイトカイン、ホルモン、免疫グロブリンなど生体内で重要な役割を果たす生理活性蛋白質は、殆どが、糖鎖の結合した糖蛋白質である。これらの糖蛋白質のポリペプチド部分は、その遺伝子がクローニングされさえすれば、遺伝子組み換え法により大量生産が可能となっている。しかし糖蛋白質の生産は、大腸菌などのような原核細胞では糖鎖が結合しないため、通常は哺乳動物細胞を用いた生産がおこなわれている。糖蛋白質の糖鎖のはたす役割は完全には解明されていないが、大腸菌により生産された糖鎖の結合しない蛋白質の研究から、糖鎖が欠失した蛋白質は生体内の半減期が、もとの糖蛋白質に比較して著しく短縮することが知られている（村上他、農化、62巻、1498-1510 、1988）。また抗体においてはＦｃ部分に結合する糖鎖は、Ｆｃ活性の発現に必須であること、造血ホルモンであるエリスロポエチンでは、in vivo の活性発現には必須であることが判明している。さらに糖鎖は、抗体の抗原特異性にも関与していることが明らかにされている。村上らは、抗体のＬ鎖に糖鎖を有するモノクローナル抗体を生産するハイブリドーマを得て、この抗原認識性を詳細に検討した結果、Ｌ鎖結合糖鎖の有無で抗原認識性が大きくかわることを確認している（Murakami H.,et al.,Animal Cell Technology:Basic & Applied Aspects, 547-551,Kluver Academic Publishers,1992)。このように生理活性糖蛋白質の活性発現には、その結合糖鎖の有無が重要な機能を果たすことが明らかとなった。

【0003】糖鎖は、真核細胞においては、細胞核内においてペプチド部分が合成され、その後、ゴルジ中で糖鎖が付加されると考えられている。糖鎖を構成する糖は、グルコース、ガラクトース、マンノース、N-アセチルグルコサミンなどのヘキソース、L-アラビノースなどのペントースなど11種類の単糖から構成されているが、これら糖種の組み合わせによって無限に近い種類の糖鎖を構成することができる。この糖鎖の結合は細胞の持つ糖転移酵素によって変化する。癌細胞など遺伝子の異常がある細胞は、これらの糖転移酵素の発現が狂い、正常

2

な細胞と異なる糖鎖が合成される。糖鎖の結合はその細胞株特有の糖鎖が結合するものとこれまでは考えられてきた。また糖鎖を結合させないようにするために、細胞内でのグリコシル化阻害剤であるツニカマイシン１０μｇ／ｍｌ程度を培地中に加えて培養することにより糖鎖の結合しない蛋白質が得られることは良く知られている。糖鎖の結合はN-グリコシド結合の場合、アスパラギン（Asn 以下アミノ酸の表記は３文字表記で示す）にN-アセチルグルコサミンが結合する。この場合、Asn-X-Ser(またはThr)(Xは任意のアミノ酸を示す）の配列を有するアミノ酸配列のAsnに糖鎖が結合する。これまでは、このN-グリコシド糖鎖の結合に関与するアミノ酸を遺伝子レベルで変換して糖鎖の結合を変えるか、あるいは遺伝子組み換えの際の宿主細胞を変えることによって糖鎖を変更することが行われてきた。後者の代表的な例としてエリスロポエチンの生産例を挙げることができる。エリスロポエチンは、分子量約34000 の糖蛋白質であり、この蛋白質は166 個のアミノ酸からなる。このアミノ酸配列をコードする遺伝子をＣＨＯ細胞とφ２細胞に導入し発現させた場合その糖鎖構造が異なることが知られている。このように宿主細胞を変えて、糖鎖構造の異なる糖蛋白質を得る方法が知られている。また糖蛋白質をグルカナーゼなどの酵素を用いて処理を行い、糖鎖を部分的に切断したりする方法も試みられているが大量生産にはあまり向いていない。糖蛋白質の糖鎖を改変して、あらたな機能を有する糖鎖を持つ糖蛋白質を得る方法は、糖鎖工学として最近新たに開発された技術であるが、まだ目的とする糖鎖を自由に得ることは出来ないのが現状である。

【0004】

【発明が解決しようとする課題】本発明者らは糖蛋白質の糖鎖構造に関する研究を進める過程において、これまで報告されてきた糖蛋白質の生産と全く異なる現象を見出した。これまでの報告によれば、糖蛋白質の糖鎖は上述したように、宿主細胞など糖蛋白質を発現する細胞に固有のものであり、癌化などの遺伝子の変異が起こらない限り糖鎖は変わらないものと考えられてきた。ところが本発明者らは、抗体生産ハイブリドーマの培養を検討したところ、細胞固有であると考えられていた糖鎖が、細胞培養条件に応じて変化してくることを見出した。本発明は、このような知見に基づいてなされたもので、動物細胞を培養し、糖蛋白質を生産する際に培地中の糖組成および／または糖濃度を変えることにより、結合糖鎖の種類あるいは分子量を改変する糖蛋白質の生産方法を提供することを課題とする。糖鎖の種類あるいは分子量を改変することにより、得られる糖蛋白質の活性や安定性の異なる物質を得ることが可能となる。

【0005】

【課題を解決するための手段】これまで動物細胞の培養にあたっては、細胞の生育に最も適した培地組成を選択

し、その培養条件を変更しないことが原則であった。従って細胞培養において最大の課題は、細胞の消費する各種成分をモニターし、それぞれの成分の減少分を補給し、最適条件を維持することであった。本発明は、細胞の生育の最適条件といわれる糖濃度、あるいは糖組成と異なる条件で、糖蛋白質を生産する動物細胞を培養するところにある。すなわち、本発明は、培地中で動物細胞を培養し糖蛋白質を生産するに当り、培地中の糖組成物および／または糖濃度を変えることにより蛋白質に結合する糖鎖の種類あるいは分子量を改変することを特徴とする糖蛋白質の製造法である。動物細胞のなかでも哺乳動物細胞はその成育条件が良く知られており、本発明を実施するに最適である。このような哺乳動物細胞としては、抗体生産ハイブリドーマ、CHO細胞、C127細胞等を挙げることができる。動物細胞を培養する場合の培養液中の糖は通常グルコースが用いられるが、まれに、培養液中の乳酸の生成を抑制する目的でフラクトースが添加される。本発明によれば、糖としてグルコースを用いる場合はその濃度を0～40mMの範囲で変化させるか、あるいはグルコースに代えて培養液中の糖質をフラクトース、マンノース、ガラクトース、N-アセチルグルコサミン、リボース、フコース、N-アセチルガラクトサミンなど、糖蛋白質の糖鎖構造中に存在する糖質に交換することで糖鎖を変換することが可能である。あるいはキトサン、デキストラン硫酸、アルギン酸など糖鎖を構成する糖などを含有する多糖を培養中に添加することで所望する効果を得ることができる。これは糖蛋白質の糖鎖を変更する方法としては、全く新しい方法である。

【0006】目的とする糖蛋白質を生産する細胞、特に遺伝子組み換えにより形質転換させた細胞株を、その細胞の生育に適した培地組成を選択して培養を行う。糖鎖の結合状態を変えるためには、最適な糖濃度よりも低い糖濃度で培養を行うことが、好ましい場合が多い。この場合の培地は無血清培地が好ましいが、血清含有培地であっても差し支えない。ハイブリドーマなど哺乳動物細胞の培養に優れた特性を示す培地として、MEM、ハムF－10培地、ハムF－12培地、RPMI1640培地、ERDF培地などが例示できるが、これらの培地に使用する最適糖濃度の0～50％濃度にすることにより、糖蛋白質の糖鎖を変更することができる。実施例に例示するように、本発明による糖鎖の変更は糖濃度による影響が大きいため、血清が生育に必須な細胞株を用いる場合には、血清を透析して糖を除去しておくことが好ましい。グルコース以外の糖質を使用する場合は、細胞の生育のための最適濃度のグルコース含量全てあるいは一部を、他の糖質に置き換えることにより、糖鎖の変更された糖蛋白質を得ることができる。とくにこのような効果を示す糖質としては、フラクトース、マンノース、ガラクトース、グルコサミン、リボース等の単糖を例示することができるが、特にガラクトース、グルコサミン、リボースの使用が好ましい。また多糖としては、キトサン、デキストラン硫酸、アルギン酸、ヘパリン、キチン、マンナンなどを例示することができ、特にキトサン、デキストラン硫酸、アルギン酸が好ましい。アルギン酸は塩であっても良いが、特にアルギン酸ナトリウムが好ましい。

【0007】細胞の培養は、細胞に適した培養方法であれば、どのような培養方法であっても使用可能である。例えば、タンクでの浮遊培養、マイクロキャリアーやウレタン素材への接着培養、ホローファイバーによる培養等が例示できる。また細胞が一定密度になるまでは、その細胞に適した従来のグルコース濃度の培養液で培養し、糖鎖を変換させた糖蛋白質を生産する段階に至って、糖の種類あるいは濃度を変更した培地に交換しても良い。多糖の場合は、多糖の溶解液を通常の培養液中に1～1000μg／mlの濃度になるように添加してもよい。培養液からの糖蛋白質は通常の精製、回収方法により分離精製できる。しかし、糖鎖の変化は糖蛋白質の分子量の差として検出できるため、SDSゲル電気泳動や、ゲル濾過方法など分子量の差による分離精製方法が適している。以下に実施例を示しさらに本発明を詳細に説明する。

【0008】

【実施例1】本実施例は、抗体生産ハイブリドーマの培養によって生産される糖蛋白質である抗体の糖鎖を変更する方法について説明する。特に本実施例により得られた糖蛋白質は糖鎖のちがいによって親和性が異なる抗体である。

（1）糖濃度、糖組成の異なる培地の調製

村上らによって開発された無血清培地であるERDF培地（特開平3－180175号公報参照）を基本培地としてこの培地のグルコース濃度を0、1、2、5、10、20、36mMの濃度にした培地を調製した。なおERDF培地のグルコース（Glc）濃度は、細胞培養時には20mMが最適とされている。またこのERDF培地の糖をフラクトース（Fru）、マンノース（Man）、ガラクトース（Gal）、N－アセチルグルコサミン（GlcNAc）、リボース（Rib）に変えた培地を調製した。さらに牛胎児血清（FCS）をリン酸緩衝生理食塩水（PBS）に対して透析を行い、グルコースなどの単糖を含まないFCS（透析FCS）を調製し、各培地に5％濃度となるように添加した。

【0009】（2）糖蛋白質の生産

糖蛋白質生産細胞として、ヒト－ヒトハイブリドーマC5TNを用いた。C5TNはハイブリドーマHB4C5(Murakami et al., In Vitro Cell. Develop.Biol., vol. 21, 593-596, 1985)の亜株でありカルボキシペプチダーゼ（以下CPA）、二本鎖DNA（以下DNA）、カンジダシトクロームC（以下CytC）に対して親和性を有するヒトIgM型抗体を分泌する細胞株である(T

5

achibana, H., et al., Biochem. Biophys. Res. Commun., vol. 189, 625-632, 1992)。このハイブリドーマが分泌する抗体（以下Ｃ５ＴＮ抗体）のＬ鎖はλ鎖であり、本発明者らは可変領域（ＶＬドメイン）のＣＤＲ１領域に-Ser-Gly-Asn-Ser-Ser-Asn-Ile-Gly- という糖鎖結合部位を持つことを明らかにしている。尚この細胞株は九州大学農学部大学院細胞工学教室から分与をうけることができる。このＣ５ＴＮを各培地５ｍｌに５×１０^{4}細胞／ｍｌの密度でまきこみ、３７℃、５％炭酸ガス雰囲気下で２４時間培養し、細胞を回収し、さらに同じ条件で４８時間培養した培養上清を回収した。

【００１０】（３）糖鎖の異なる糖蛋白質の分離

6

＊各培地で培養した培養上清を還元条件下でＳＤＳ－ポリアクリルアミドゲル電気泳動（ＳＤＳ－ＰＡＧＥ）を行った。各λ鎖は、抗λ抗体を用いたウエスタンブロット法により確認を行った（図１、図２）。Ｌ鎖は４種類検出されたが、泳動の位置から判断して分子量の大きいほうからＬ１、Ｌ２、Ｌ３、Ｌ４と名付けた。また糖鎖合成阻害剤であるツニカマイシン存在下ではＬ４のみが合成された。Ｌ４は糖鎖の結合しない抗体であることが推定された。各培地での抗体の生産を表１に示した。

【００１１】

【表１】

培地糖組成	(mM)	Ｌ鎖のタイプ
Ｇｌｃ	0	Ｌ１，Ｌ３，Ｌ４
Ｇｌｃ	1	Ｌ１，Ｌ３，Ｌ４
Ｇｌｃ	2	Ｌ１，Ｌ３，Ｌ４
Ｇｌｃ	5	Ｌ１，Ｌ２，Ｌ３，Ｌ４
Ｇｌｃ	１０	Ｌ１，Ｌ２
Ｇｌｃ	２０	Ｌ１，Ｌ２
Ｇｌｃ	３６	Ｌ１，Ｌ２
Ｆｒｕ	２０	Ｌ１，Ｌ２
Ｍａｎ	２０	Ｌ１，Ｌ２
Ｇａｌ	２０	Ｌ１，Ｌ２，Ｌ４
ＧｌｃＮＡｃ	２０	Ｌ１，Ｌ３，Ｌ４
Ｒｉｂ	２０	Ｌ１，Ｌ３，Ｌ４

【００１２】培地中のグルコース濃度、あるいは糖質の変化により通常の培養条件では得られないＬ２の抗体を得ることができた。この抗体はＳＤＳ－ＰＡＧＥにより他の抗体から分離することができた。本細胞は上述したように４種類の分子量を持つλ鎖（Ｌ鎖）を含む抗体を生産しており、このλ鎖（Ｌ鎖）分子量の差は、糖鎖の分子量差によるものである。

【００１３】

【実施例２】

糖鎖の違いによる抗体の特性変化

Ｃ５ＴＮ抗体はＬ鎖に糖鎖が結合しており、本発明によりこの糖鎖を（３）のような分子量の異なる糖鎖とすることができた。この糖鎖の変更によって抗体特異性が変化することを以下のように確認した。抗体の抗原特異性は、抗原をコートしたマイクロプレートを用いた酵素免疫測定法（ＥＬＩＳＡ）で測定できる（H.Murakami et al.,Animal Cell Technology:Basic & Applied Aspects,547-551,1992,Kluwer Acdemic Publishers)。各培地で培養したＣ４ＴＮ抗体２００ｎｇ／ｍｌの溶液を調製し、この抗体溶液の抗原特異性を測定した。測定値は、Ｇｌｃ　２０ｍＭで培養して得た抗体の反応性を１００％とした百分率で表した。結果を図３および図４に示した。Ｇｌｃの含量を変化させた場合、Ｌ３、Ｌ４の増加とともにＣＰＡ親和性およびＣｙｔｃ親和性は低下したが、ＤＮＡ親和性は２ｍＭでピークに達した。また培地中の糖組成を変えた場合は、抗体の親和性はＧａｌ、ＧｌｃＮＡｃ、Ｒｉｂ添加により、Ｇｌｃで培養して得た抗体と全く異なる結果を示した（図３、図４）。これは、抗体に結合する糖鎖の構造が変化したためと推定された。

【００１４】

【実施例３】グルコース濃度を１０ｍＭに調整したＥＲＤＦ基本無血清培地を用いて、実施例１と同様の条件でハイブリドーマＣ５ＴＮ細胞の培養を行った。すなわち、ハイブリドーマＣ５ＴＮを５×１０^{4}細胞／mlの密度でまきこみ、６時間培養を行った。その後細胞を回収し、キトサン添加（１μｇ／ｍｌ）、デキストラン硫酸添加（１００μｇ／ml）あるいはアルギン酸添加（１００μｇ／ｍｌ）培養液（ＥＲＤＦ）に移し、さらに48時間培養を行った後、培養上清を回収し、これを透析濃縮した。培養条件は３７℃、５％CO_2 雰囲気下で実施例１と同様におこなった。又対照は、ＥＲＤＦのみで培養をおこなった。この濃縮液を還元条件でＳＤＳ－ＰＡＧＥにより電気泳動を行い、抗ヒトλＬ鎖抗体で、ウエス

7

タンブロット法により抗体のＬ鎖を特異的に検出した。結果を図５に示した。各多糖類を添加することにより、グルコースのみの培養と比較して２８ＫＤおよび３０ＫＤのＬ鎖が増加した。これは抗体の糖鎖が変化していることを裏付ける結果であった。また得られた抗体はいずれもＩｇＭ型の抗体のみであった。

【００１５】

【実施例４】実施例３で生産された糖鎖が変化した抗体の抗原親和性の変化を、実施例２に開示した方法により確認した。抗原をコートしたマイクロプレートを用いて同様に測定を行い、ＥＬＩＳＡ法により各抗原に対する反応性を測定した。各抗原に対する反応性はＥＬＩＳＡ法による発色の変化を４０５ｎｍの吸光度変化により観察した。結果を図６～８に示した。抗体は糖鎖の変化により、抗原との反応性が変化していることが確認された。

【００１６】

【発明の効果】本発明により、動物細胞を培養して糖蛋白質を生産する際に、培地中のリボース、ガラクトース、グルコサミン等の単糖の組成あるいは糖濃度等の培養条件を制御することにより、蛋白質に付加する糖鎖の種類あるいは分子量を改変し、特性の変化した糖蛋白質を生産することができる。また培地中にアルギン酸、デキストラン硫酸、キトサンのような多糖を添加して糖鎖の種類、あるいは分子量を改変し、特性の変化した糖蛋白質を生産することができる。この方法により糖鎖の種類あるいは分子量の異なる糖蛋白質を得ることができる。また糖鎖の改変によって糖蛋白質の生物活性をも変更することができる。

【図面の簡単な説明】

【図１】実施例１により、グルコース濃度を変えて培養

8

したＣ５ＴＮ細胞より得られるλ型抗体のＳＤＳ－ＰＡＧＥウエスタンブロットによる検出パターンを示す。

【図２】実施例１により、グルコースを他の糖質に変えて培養したＣ５ＴＮ細胞より得られるλ型抗体のＳＤＳ－ＰＡＧＥウエスタンブロットによる検出パターンを示す。

【図３】実施例２により、グルコース濃度を変えて培養したＣ５ＴＮ細胞より得られる抗体の抗原親和性の変化を示す。

【図４】実施例２により、グルコースを他の糖質に変えて培養したＣ５ＴＮ細胞より得られる抗体の抗原親和性を示す。

【図５】実施例３より得たヒトλ型抗体のＳＤＳ－ＰＡＧＥウエスタンブロットによる検出パターンを示す。

【符号の説明】

レーン１：分子サイズマーカー
レーン２：対照
レーン３：キトサン添加（１μｇ／ml）
レーン４：デキストラン硫酸添加（100 μｇ／ml）
レーン５：アルギン酸添加（100 μｇ／ml）

【図６】実施例３により得られた抗体のＣＰＡに対する親和性の変化を示す。

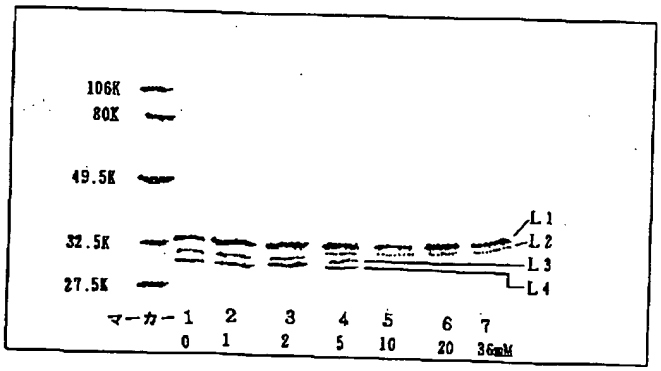
【図７】実施例３により得られた抗体のＣｙｔＣに対する親和性の変化を示す。

【図８】実施例３により得られた抗体のｄｓＤＮＡに対する親和性の変化を示す。

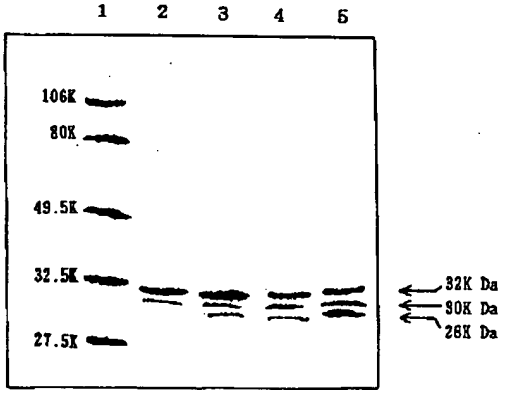
【符号の説明】

－○－　対照
－□－　キトサン添加（１μｇ／ml）
－●－　デキストラン硫酸添加（100 μｇ／ml）
－△－　アルギン酸添加（100 μｇ／ml）

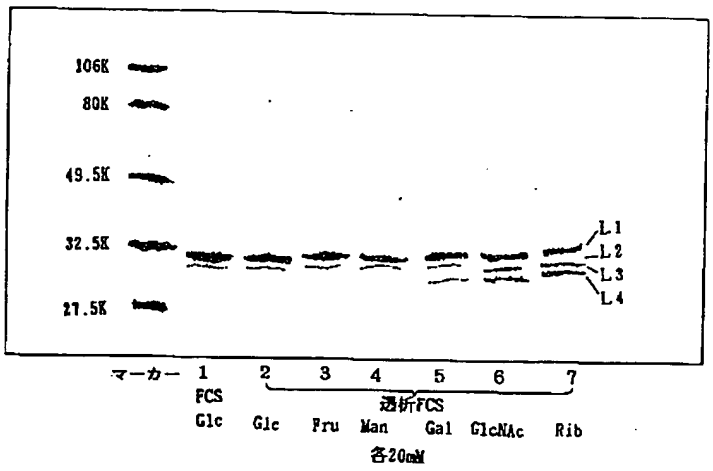
【図１】



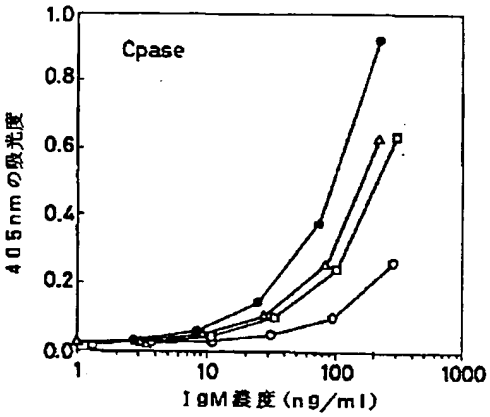
【図５】



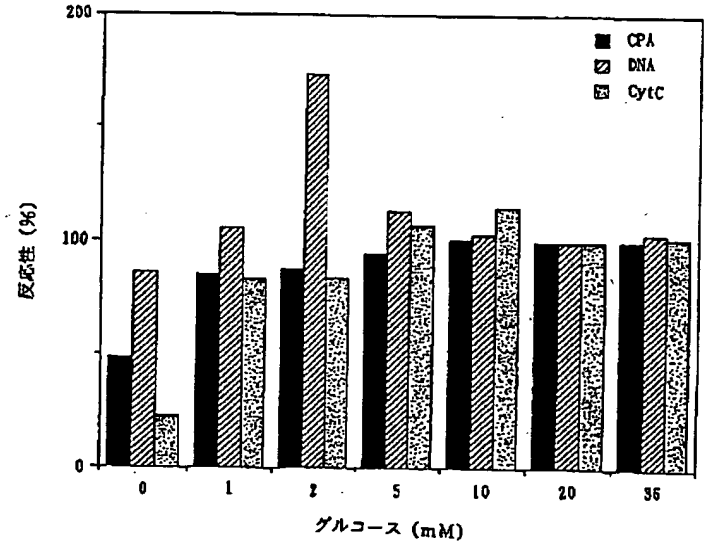
【図2】


106K
80K
49.5K
32.5K
27.5K
L1
L2
L3
L4
マーカー 1 2 3 4 5 6 7
FCS
Glc
透析FCS
Glc Fru Man Gal GlcNAc Rib
各20mM

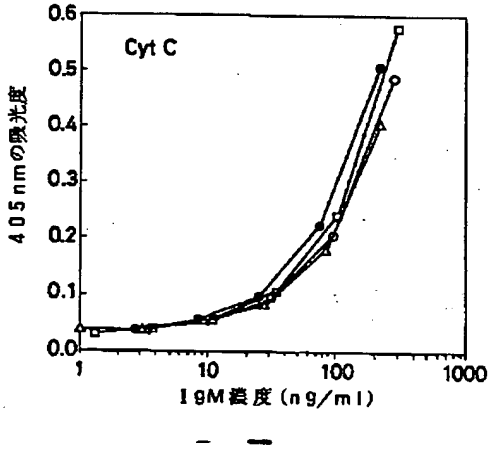
【図6】


Cpase
405nmの吸光度
IgM濃度(ng/ml)

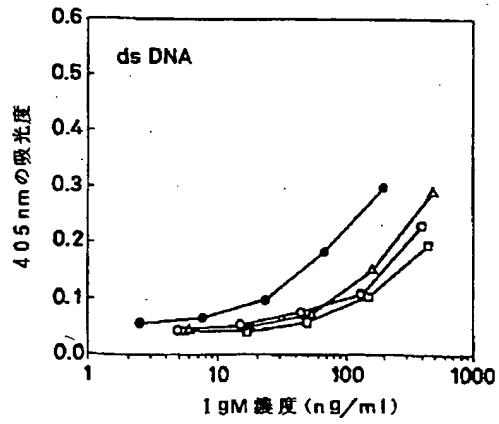
【図3】


CPA
DNA
CytC
反応性(%)
グルコース(mM)

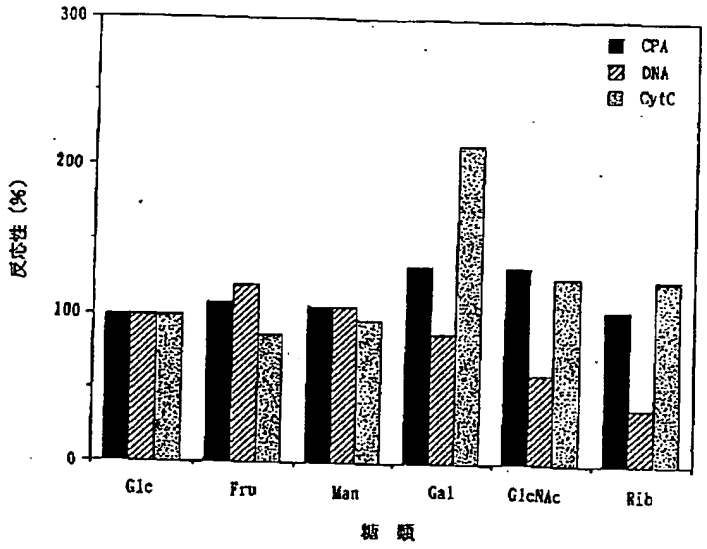
【図7】


Cyt C
405nmの吸光度
IgM濃度(ng/ml)

【図8】


ds DNA
405nmの吸光度
IgM濃度(ng/ml)

【図4】



フロントページの続き

(51)Int.Cl.[5]	識別記号	庁内整理番号	FI	技術表示箇所
C12R 1:91)				

An abridged translation of Cited Document 1

09/535533

Cited Document 1: JP-A Publication No. Hei 06-292592

Column 3, lines 18-27:

According to the present invention, the concentration of glucose, if used as a sugar source, may be varied from 0-40 mM, or as the sugar source in a culture medium, glucose can be replaced with other carbohydrates, such as fructose, mannose, galactose, N-acetylglucosamine, ribose, fucose and N-acetylgalactosamine, which are present in the sugar chain structures present in the glycoprotein. Alternatively, polysaccharides, such as chitosan, dextran sulfate and alginate, which contain sugars serving as components for sugar chains may be added to the medium during the culture to obtain a desired effect.

English abstract of Cited Document 2

PatentWeb Home | Edit Search | Return to Patent List | Help

Include in patent order

MicroPatent(R) Worldwide PatSearch: Record 1 of 1

[no drawing available]

Family Lookup

JP04281797

METHOD FOR PREPARING CULTURE MEDIUM FOR PRODUCING MONOCLONAL ANTIBODY AND ITS KIT

MORINAGA & CO LTD

Inventor(s): ;MOCHIZUKI KATSUMI ;SATO SUSUMU ;HASHIZUME SHUICHI

Application No. 03069271 , Filed 19910308 , Published 19921007

Abstract:

PURPOSE: To provide a method for preparing a culture medium in which the production of a monoclonal antibody can be enhanced and obtain a kit for preparing the culture medium used for the aforementioned method.

CONSTITUTION: A glucide and glutamine are added at various concentrations to a culture medium without containing the glucide and glutamine to prepare culture media. The resultant respective culture media are used to culture a cell capable of producing an antibody. Thereby, the concentrations of the glucide and the glutamine at which the production of the antibody is most enhanced are set. If a kit composed of a culture medium without containing the glucide and glutamine, the glucide and the glutamine is used, culture media at variously changed concentrations of the glucide and glutamine can readily be prepared. Fructose is especially preferably used as the glucide.

Int'l Class: C12P02108 C12N00516 C12N01507 C12P02108 C12R00191

MicroPatent Reference Number: 001388802

PatentWeb Home | Edit Search | Return to Patent List | Help

For further information, please contact:
Technical Support | Billing | Sales | General Information

XP-002085768

1/1 - (C) WPI / DERWENT
AN - 95-009082 ç02!
AP - JP940031019 940202
PR - JP930044587 930209
TI - Prodn. of glyco-protein(s) having different activities - by culturing animal cells in medium with varied sugar compsn. and/or concn.
IW - PRODUCE GLYCO PROTEIN ACTIVE CULTURE ANIMAL CELL MEDIUM VARY SUGAR COMPOSITION CONCENTRATE
PA - (SNOW) SNOW BRAND MILK PROD CO LTD
PN - JP6292592 A 941021 DW9502 C12P21/00 007pp
ORD - 1994-10-21
IC - C12P21/08
FS - CPI
DC - B04 D16
AB - J06292592 Prodn. is effected by culturing animal cells in a medium to produce glycoprotein in medium with varied sugar compsn. and/or sugar concn. so that types or molecular wts. of sugar chains which combine with protein may be modified.
- USE/ADVANTAGE - Different glycoproteins having different activities and stabilities with modified sugar chains may be produced under different culture conditions. Culture conditions: culture condition is pref. changed by adding monosaccharides e.g. ribose, galactose, and glucosamine or polysaccharides e.g. arginic acid, dextran sulphate, and chitosan in different concn. in a medium.
- In an example, mediums were prepd. by adding glucose of 0, 1, 2, 5, 10, 20 and 36mM concns. to ERDF medium (Jap. Pat. Disclosure No 180175/91) or replacing sugars in ERDF medium with fructose, mannose, galactose, N-acetylglycosamine, or ribose. Then human/human hybridoma C5TN cells which is subclass of hybridoma HB4C5 and secrets human Ig M antibody were cultured at 50000 cells/ml at 5% CO2 at 37 deg.C for 24 hrs. Then, the supernatant soln. was sepd. The cells were cultured for 48 hrs. in the same conditions to separate the supernatant soln. The Ig M antibody has a region for combining sugar chain (-Ser-Gly-Asn-Ser-Sr-Asn-Ile-Gl y-) in CDR1 region of VL domain. Each supernatant soln. was subjected to SDS-PAGE to detect 4L chains of different mol. wt. The mol. wt. differences are derived from differences of mol. wt. of sugar chains. (Dwg.0/5)

TECHNICAL FIELD

[Industrial Application] In case this invention cultivates an animal cell in a culture medium and produces glycoprotein, by controlling the culture condition, especially culture-medium conditions of a cell, it changes the class or molecular weight of a sugar chain combined with protein, and relates to the approach of producing the glycoprotein from which the property changed.

PRIOR ART

[Description of the Prior Art] Most of the bioactive protein which plays important roles, such as lymphokine, cytokine, hormone, and an immunoglobulin, in the living body is glycoprotein which the sugar chain combined. The polypeptide part of these glycoprotein can be mass-produced by gene recombination, if only cloning of the gene is carried out. However, in order that a sugar chain may not combine production of glycoprotein by prokaryotic cells, such as Escherichia coli, production using a mammalian cell is usually performed. Although the role which the sugar chain of glycoprotein plays is not solved thoroughly, it is known from research of the protein which the sugar chain produced with Escherichia coli does not combine that a half-life in the living body will shorten remarkably the protein in which the sugar chain carried out deletion as compared with the glycoprotein of a basis (**-izing besides Murakami, 62 volumes, 1498-1510, 1988). . Moreover, the sugar chain combined with Fc part in an antibody is in vivo at the erythropoietin which is indispensable to the manifestation of Fc activity, and hematogenous hormone. The indispensable thing has become clear for the activity manifestation. Furthermore, it is shown clearly that the sugar chain is participating also in the antigenic specificity of an antibody. Murakami and others is checking that antigen recognition nature changes greatly by the existence of an L chain joint sugar chain, as a result of obtaining the hybridoma which produces the monoclonal antibody which has a sugar chain in the L chain of an antibody and examining this antigen recognition nature in a detail (Murakami H., et al., Animal Cell Technology:Basic & Applied Aspects, 547-551, Kluver Academic Publishers, 1992). Thus, it became clear to achieve a function with the existence of the joint sugar chain important for the activity manifestation of bioactive glycoprotein.
[0003] In the eukaryotic cell, a peptide part is compounded in a nucleus and the sugar chain is considered that a sugar chain is added in Golgi after that. Although the sugar which constitutes a sugar chain consists of 11 kinds of monosaccharides, such as pentoses, such as hexoses, such as a glucose, a galactose, a mannose, and N-acetyl glucosamine, and L-arabinose, it can constitute the sugar chain of the class near infinity with the combination of these ****. Association of this sugar chain changes with the glycosyltransferases which a cell has. As for the cell with the abnormalities of genes, such as a cancer cell, the sugar chain with which the manifestation of these glycosyltransferases differs from deviation and a normal cell is compounded. It has been thought until now that a sugar chain peculiar to the cell strain combines association of a sugar chain. Moreover, in order to make it not combine a sugar chain, it is well known by adding about [which is an intracellular glycosylation inhibitor] tunicamycin 10microg/ml into a culture medium, and cultivating it that the protein which a sugar chain does not combine will be obtained. In the case of N-glycosidic linkage, association of a sugar chain is an asparagine (Asn a three-character notation shows the notation of amino acid below). N-acetyl glucosamine joins together. In this case, Asn-X-Ser (or Thr) (X shows the amino acid of arbitration) A sugar chain combines with Asn of an amino acid sequence which has an array. Until now, changing a sugar chain has been performed by changing the amino acid which participates in association of this N-glycoside sugar chain on gene level, and changing association of a sugar chain, or changing the host cell in the case of transgenics. The example of production of erythropoietin can be given as a latter typical example. Erythropoietin is molecular weight 34000 [about]. It is glycoprotein and this protein is 166. It consists of amino acid of an individual. When a CHO cell and phi2 cell are made to introduce and discover the gene which carries out the code of this amino acid sequence, it is known that those sugar chain structures differ. Thus, a host cell is changed and the method of obtaining the glycoprotein with which sugar chain structures differ is learned. Moreover, glycoprotein is processed using enzymes, such as a glucanase, and although the approach which has cut the sugar chain enough selectively and carries out it is also tried, it is seldom fit for mass production method. Although the method of obtaining glycoprotein with the sugar chain which changes the sugar chain of glycoprotein and has a new function is the technique newly developed as glycoengineering recently, the actual condition is being unable to obtain the target sugar chain freely yet.

EFFECT OF THE INVENTION

[Effect of the Invention] In case an animal cell is cultivated and glycoprotein is produced by this invention, by controlling culture conditions, such as a presentation of monosaccharides, such as a ribose in a culture medium, a galactose, and a glucosamine, or sugar concentration, the class or molecular weight of a sugar chain added to protein can be changed, and the glycoprotein from which the property changed can be produced. Moreover, an alginic acid, dextran sulfate, and a polysaccharide like chitosan can be added in a culture medium, the class of sugar chain or molecular weight can be changed, and the glycoprotein from which the property changed can be produced. The glycoprotein from which the class of sugar chain or molecular weight differs by this approach can be obtained. Moreover, the bioactive of glycoprotein can also be changed by the alteration of a sugar chain.

TECHNICAL PROBLEM

[Problem(s) to be Solved by the Invention] this invention persons found out a completely different phenomenon from production of the glycoprotein reported so far in the process in which research on the sugar chain structure of glycoprotein is advanced. According to the old report, as the sugar chain of glycoprotein was mentioned above, unless it is the thing of a proper and the variation of genes, such as canceration, takes place to the cell which discovers glycoprotein, such as a host cell, it has been thought that a sugar chain does not change. However, when this invention persons considered culture of an antibody production hybridoma, they found out that the sugar chain considered to be peculiar to a cell changed according to cell culture conditions. In case this invention was made based on such knowledge, cultivates an animal cell and produces glycoprotein, it makes it a technical problem to offer the process of glycoprotein which changes the class or molecular weight of a joint sugar chain by changing the sugar composition and/or sugar concentration in a culture medium. By changing the class or molecular weight of a sugar chain, it becomes possible to obtain the matter with which the activity of the glycoprotein obtained differs from stability.

[Translation done.]

MEANS

[Means for Solving the Problem] It was a principle to choose the medium composition which was most suitable for growth of a cell, and not to change the culture condition in culture of an animal cell, until now. Therefore, the biggest technical problem in a cell culture was acting as the monitor of the various components which a cell's consumes, supplying the decrement of each component, and maintaining optimum conditions. This invention is the sugar concentration called optimum conditions of growth of a cell, or different conditions from sugar composition, and is in the place which cultivates the animal cell which produces glycoprotein. That is, in cultivating an animal cell in a culture medium and producing glycoprotein, this invention is the manufacturing method of the glycoprotein characterized by changing the class or molecular weight of a sugar chain combined with protein by changing the sugar composition object and/or sugar concentration in a culture medium. The mammalian cell is the the best for the growth condition being known well and carrying out this invention also in an animal cell. As such a mammalian cell, an antibody production hybridoma, a CHO cell, C127 cell, etc. can be mentioned. Fructose is added in order for the sugar in the culture medium in the case of cultivating an animal cell to control generation of the lactic acid in culture medium rarely, although a glucose is usually used. According to this invention, when using a glucose as sugar, it is possible to change a sugar chain by changing the concentration in the range of 0-40mM, or replacing with a glucose, and exchanging the sugar in culture medium for the sugar which exists in sugar chain structures of glycoprotein, such as fructose, a mannose, a galactose, N-acetyl glucosamine, a ribose, fucose, and N-acetyl galactosamine. Or the effectiveness for which it asks by adding while cultivating the polysaccharide containing the sugar which constitutes sugar chains, such as chitosan, dextran sulfate, and an alginic acid, can be acquired. As an approach of changing the sugar chain of glycoprotein, this is a completely new approach.

[0006] It cultivates by choosing the medium composition which was suitable for growth of the cell in the cell which produces target glycoprotein, especially the cell strain which carried out the transformation by transgenics. In order to change the integrated state of a sugar chain, it is desirable to cultivate by sugar concentration lower than the optimal sugar concentration in many cases. Although the culture medium in this case has a desirable serum free medium, it does not interfere, even if it is a blood serum content culture medium. As a culture medium which shows the property excellent in culture of mammalian cells, such as a hybridoma, although MEM, F-hum 10 culture medium, F-hum 12 culture medium, RPMI1640 culture medium, an ERDF culture medium, etc. can be illustrated, the sugar chain of glycoprotein can be changed by making it 0 - 50% concentration of the optimal sugar concentration used for these culture media. Since the effect by sugar concentration is large, when a blood serum uses an indispensable cell strain for growth, it is desirable [modification of the sugar chain by this invention] to dialyze a blood serum and to remove sugar, so that it may illustrate in the example. When using sugar other than a glucose, the glycoprotein with which the sugar chain was changed can be obtained by transposing to other sugar for [of optimum density / all glucose contents or a part of] growth of a cell. As sugar which shows such effectiveness especially, although monosaccharides, such as fructose, a mannose, a galactose, a glucosamine, and a ribose, can be illustrated, the activity of a galactose, a glucosamine, and a ribose is especially desirable. Moreover, as a polysaccharide, chitosan, dextran sulfate, an alginic acid, heparin, a chitin, a mannan, etc. can be illustrated, and especially chitosan, dextran sulfate, and an alginic acid are desirable. Although an alginic acid may be a salt, especially sodium alginate is desirable.

[0007] If it is the culture approach suitable for a cell, no matter culture of a cell may be what culture approach, it is usable. For example, the suspended cell culture in a tank, the adhesion culture to a micro carrier or an urethane raw material, culture by the hollow fiber, etc. can be illustrated. Moreover, it may cultivate with the culture medium of the conventional glucose concentration suitable for the cell, and you may exchange for the culture medium which changed the class or concentration of sugar into the phase of producing the glycoprotein to which the sugar chain was transformed, very much until a cell becomes a fixed consistency. In the case of a polysaccharide, the solution of a polysaccharide may be

added so that it may become 1-1000microg [/ml] concentration into the usual culture medium. The glycoprotein from culture medium can carry out separation purification by the usual purification and the recovery approach. However, since change of a sugar chain is detectable as a difference of the molecular weight of glycoprotein, SDS-gel electrophoresis and the separation purification approaches by the difference of molecular weight, such as the gel filtration approach, are suitable. An example is shown below and this invention is further explained to a detail.
[0008]
[Example 1] This example explains how to change the sugar chain of the antibody which is glycoprotein produced by culture of an antibody production hybridoma. The glycoprotein obtained by especially this example is an antibody from which compatibility differs by the difference in a sugar chain.
(1) The culture medium which made glucose concentration of this culture medium the concentration of 0, 1, 2, 5, 10, 20, and 36mM by making into a basal medium the ERDF culture medium (referring to JP,3-180175,A) which is a serum free medium developed by preparation Murakami and others of a culture medium where sugar concentration differs from sugar composition was prepared. In addition, glucose (Glc) concentration of an ERDF culture medium is made the optimal [20mM(s)] at the time of a cell culture. Moreover, the culture medium which changed the sugar of this ERDF culture medium into fructose (Fru), a mannose (Man), a galactose (Gal), N-acetyl glucosamine (GlcNAc), and a ribose (Rib) was prepared. Furthermore, fetal calf serum (FCS) was dialyzed to phosphate buffered saline (PBS), and FCS (dialysis FCS) which does not contain monosaccharides, such as a glucose, was prepared, and it added so that it might become concentration 5% at each culture medium.
[0009] (2) Homo sapiens-Homo sapiens hybridoma C5TN was used as a production glycoprotein production cell of glycoprotein. C5TN is the substrain of hybridoma HB4C5 (Murakami et al., In Vitro Cell.Develop.Biol., vol.21, 593-596, 1985). Carboxypeptidase (henceforth, CPA), Double stranded DNA (henceforth, DNA), As opposed to Candida citchrome C (henceforth, CytC) It is the cell strain which secretes the Homo sapiens IgM mold antibody which has compatibility (Tachibana, H., et al., Biochem.Biophys.Res.Commun., vol.189, 625-632, 1992). the L chain of the antibody (following C5TN antibody) which this hybridoma secretes -- a lambda chain -- it is -- this invention persons -- CDR1 field of a variable region (VL domain) -Ser-Gly-Asn-Ser-Ser-Asn-Ile-Gly- ** -- it is shown clearly that it has the sugar chain binding site to say. In addition, this cell strain can receive distribution from the Kyushu University agricultural department graduate school cell technology classroom. It is this C5TN in 5ml of each culture medium 5x104 It wound, was crowded with the cell / consistency of ml, and cultivated for 24 hours under 37 degrees C and 5% carbon-dioxide-gas ambient atmosphere, cells were collected, and the culture supernatants cultivated on the still more nearly same conditions for 48 hours were collected.
[0010] (3) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the culture supernatant cultivated by separation each culture medium of the glycoprotein with which sugar chains differ under reduction conditions. Each lambda chain checked by the western blotting which used the ** lambda antibody (drawing 1 , drawing 2). Although four kinds of L chains were detected, they named L1, L2, L3, and L4 from the one where molecular weight is larger, judging from the location of migration. Moreover, only L4 was compounded under the tunicamycin existence which is a sugar chain composition inhibitor. It was presumed that L4 is an antibody which a sugar chain does not combine. Production of the antibody in each culture medium was shown in a table 1.
[0011]
[A table 1]
---------- Culture-medium sugar composition (mM) The type of an L chain ---------- ---------- Glc 0 L1, L3, L4 Glc 1 L1, L3, L4 Glc 2 L1, L3, L4 Glc 5 L1, L2, L3, L4 Glc 10 L1, L2 Glc 20 L1, L2 Glc 36L1, L2 Fru20 L1, L2 Man 20 L1, L2 Gal 20 L1, L2, L4GlcNAc 20L1, L3, L4 Rib 20 L1, L3, L4 ---------- [0012] The glucose concentration in a culture medium or the antibody of L2 which is not obtained by the usual culture condition by change of sugar was able to be obtained. This antibody was separable from other antibodies with SDS-PAGE. This cell produces the antibody containing a lambda chain (L chain) with four kinds of molecular weight, as mentioned above, and the difference of this lambda chain (L chain) molecular weight is based on the molecular weight

difference of a sugar chain.
[0013]
[Example 2]
The sugar chain had combined with the L chain and the property change C5TN antibody of the antibody by the difference in a sugar chain was able to be used as the sugar chain from which molecular weight as shown in (3) differs this sugar chain by this invention. It checked as follows that antibody specificity changed with modification of this sugar chain. The antigenic specificity of an antibody can measure an antigen with the enzyme immunoassay (ELISA) using the microplate which carried out the coat (H. Murakami et al., Animal Cell Technology:Basic & Applied Aspects, 547- 551, 1992, Kluwer Acdemic Publishers). The solution with a C4TN antibody of 200 ng/ml cultivated by each culture medium was prepared, and the antigenic specificity of this antibody solution was measured. Measured value is Glc. The reactivity of the antibody cultivated and obtained by 20mM was expressed with the percentage made into 100%. The result was shown in drawing 3 and drawing 4 . Although CPA compatibility and Cytc compatibility fell with the increment in L3 and L4 when changing the content of Glc, DNA compatibility reached the peak in 2mM(s). Moreover, when the sugar composition in a culture medium was changed, the compatibility of an antibody showed a completely different result from the antibody cultivated and obtained by Glc by Gal, GlcNAc, and Rib addition (drawing 3 , drawing 4). Since the structure of the sugar chain combined with an antibody changed, this was presumed.
[0014]
[Example 3] The hybridoma C5TN cell was cultivated on the same conditions as an example 1 using the ERDF basic serum free medium which adjusted glucose concentration to 10mM(s). That is, it is hybridoma C5TN 5x104 It wound, and was crowded with the cell / consistency of ml, and culture was performed for 6 hours. After having collected cells after that, moving to chitosan addition (1microg/(ml)), dextran sulfate addition (100microg/(ml)), or alginic-acid addition (100microg/(ml)) culture medium (ERDF) and performing culture for further 48 hours, culture supernatants were collected and dialysis concentration of this was carried out. A culture condition is 37 degrees C and 5%CO2. It carried out like the example 1 under the ambient atmosphere. Moreover, contrast cultivated only by ERDF. SDS-PAGE performed electrophoresis for this concentration liquid on reduction conditions, and the L chain of an antibody was specifically detected by western blotting by the anti-Homo sapiens lambda L chain antibody. The result was shown in drawing 5 . By adding each polysaccharide, the L chain of 28KD(s) and 30KD(s) increased as compared with culture of only a glucose. This was the result of proving that the sugar chain of an antibody is changing. Moreover, each obtained antibody was only an antibody of an IgM mold.
[0015]
[Example 4] Change of the antigen compatibility of the antibody from which the sugar chain produced in the example 3 changed was checked by the approach indicated in the example 2. It measured similarly using the microplate which carried out the coat of the antigen, and the reactivity over each antigen was measured by the ELISA method. The reactivity over each antigen observed change of coloring by the ELISA method by 405nm absorbance change. The result was shown in drawing 6 -8. As for the antibody, it was checked by change of a sugar chain that reactivity with an antigen is changing.

[Translation done.]

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application] In case this invention cultivates an animal cell in a culture medium and produces glycoprotein, by controlling the culture condition, especially culture-medium conditions of a cell, it changes the class or molecular weight of a sugar chain combined with protein, and relates to the approach of producing the glycoprotein from which the property changed.
[0002]
[Description of the Prior Art] Most of the bioactive protein which plays important roles, such as lymphokine, cytokine, hormone, and an immunoglobulin, in the living body is glycoprotein which the sugar chain combined. The polypeptide part of these glycoprotein can be mass-produced by gene recombination, if only cloning of the gene is carried out. However, in order that a sugar chain may not combine production of glycoprotein by prokaryotic cells, such as Escherichia coli, production using a mammalian cell is usually performed. Although the role which the sugar chain of glycoprotein plays is not solved thoroughly, it is known from research of the protein which the sugar chain produced with Escherichia coli does not combine that a half-life in the living body will shorten remarkably the protein in which the sugar chain carried out deletion as compared with the glycoprotein of a basis (**-izing besides Murakami, 62 volumes, 1498-1510, 1988). . Moreover, the sugar chain combined with Fc part in an antibody is in vivo at the erythropoietin which is indispensable to the manifestation of Fc activity, and hematogenous hormone. The indispensable thing has become clear for the activity manifestation. Furthermore, it is shown clearly that the sugar chain is participating also in the antigenic specificity of an antibody. Murakami and others is checking that antigen recognition nature changes greatly by the existence of an L chain joint sugar chain, as a result of obtaining the hybridoma which produces the monoclonal antibody which has a sugar chain in the L chain of an antibody and examining this antigen recognition nature in a detail (Murakami H., et al., Animal Cell Technology:Basic & Applied Aspects, 547-551, Kluver Academic Publishers, 1992). Thus, it became clear to achieve a function with the existence of the joint sugar chain important for the activity manifestation of bioactive glycoprotein.
[0003] In the eukaryotic cell, a peptide part is compounded in a nucleus and the sugar chain is considered that a sugar chain is added in Golgi after that. Although the sugar which constitutes a sugar chain consists of 11 kinds of monosaccharides, such as pentoses, such as hexoses, such as a glucose, a galactose, a mannose, and N-acetyl glucosamine, and L-arabinose, it can constitute the sugar chain of the class near infinity with the combination of these ****. Association of this sugar chain changes with the glycosyltransferases which a cell has. As for the cell with the abnormalities of genes, such as a cancer cell, the sugar chain with which the manifestation of these glycosyltransferases differs from deviation and a normal cell is compounded. It has been thought until now that a sugar chain peculiar to the cell strain combines association of a sugar chain. Moreover, in order to make it not combine a sugar chain, it is well known by adding about [which is an intracellular glycosylation inhibitor] tunicamycin 10microg/ml into a culture medium, and cultivating it that the protein which a sugar chain does not combine will be obtained. In the case of N-glycosidic linkage, association of a sugar chain is an asparagine (Asn a three-character notation shows the notation of amino acid below). N-acetyl glucosamine joins together. In this case, Asn-X-Ser (or Thr) (X shows the amino acid of arbitration) A sugar chain combines with Asn of an amino acid sequence which has an array. Until now, changing a sugar chain has been performed by changing the amino acid which participates in association of this N-glycoside sugar chain on gene level, and changing association of a sugar chain, or changing the host cell in the case of transgenics. The example of production of erythropoietin can be given as a latter typical example. Erythropoietin is molecular weight 34000 [about]. It is glycoprotein and this protein is 166. It consists of amino acid of an individual. When a CHO cell and phi2 cell are made to introduce and discover the gene which carries out the code of this amino acid sequence, it is known that those sugar chain structures differ. Thus, a host cell is changed and the method of obtaining the glycoprotein with which sugar chain structures differ is learned. Moreover, glycoprotein is processed using enzymes, such

as a glucanase, and although the approach which has cut the sugar chain enough selectively and carries out it is also tried, it is seldom fit for mass production method. Although the method of obtaining glycoprotein with the sugar chain which changes the sugar chain of glycoprotein and has a new function is the technique newly developed as glycoengineering recently, the actual condition is being unable to obtain the target sugar chain freely yet.

[0004]

[Problem(s) to be Solved by the Invention] this invention persons found out a completely different phenomenon from production of the glycoprotein reported so far in the process in which research on the sugar chain structure of glycoprotein is advanced. According to the old report, as the sugar chain of glycoprotein was mentioned above, unless it is the thing of a proper and the variation of genes, such as canceration, takes place to the cell which discovers glycoprotein, such as a host cell, it has been thought that a sugar chain does not change. However, when this invention persons considered culture of an antibody production hybridoma, they found out that the sugar chain considered to be peculiar to a cell changed according to cell culture conditions. In case this invention was made based on such knowledge, cultivates an animal cell and produces glycoprotein, it makes it a technical problem to offer the process of glycoprotein which changes the class or molecular weight of a joint sugar chain by changing the sugar composition and/or sugar concentration in a culture medium. By changing the class or molecular weight of a sugar chain, it becomes possible to obtain the matter with which the activity of the glycoprotein obtained differs from stability.

[0005]

[Means for Solving the Problem] It was a principle to choose the medium composition which was most suitable for growth of a cell, and not to change the culture condition in culture of an animal cell, until now. Therefore, the biggest technical problem in a cell culture was acting as the monitor of the various components which a cell's consumes, supplying the decrement of each component, and maintaining optimum conditions. This invention is the sugar concentration called optimum conditions of growth of a cell, or different conditions from sugar composition, and is in the place which cultivates the animal cell which produces glycoprotein. That is, in cultivating an animal cell in a culture medium and producing glycoprotein, this invention is the manufacturing method of the glycoprotein characterized by changing the class or molecular weight of a sugar chain combined with protein by changing the sugar composition object and/or sugar concentration in a culture medium. The mammalian cell is the the best for the growth condition being known well and carrying out this invention also in an animal cell. As such a mammalian cell, an antibody production hybridoma, a CHO cell, C127 cell, etc. can be mentioned. Fructose is added in order for the sugar in the culture medium in the case of cultivating an animal cell to control generation of the lactic acid in culture medium rarely, although a glucose is usually used. According to this invention, when using a glucose as sugar, it is possible to change a sugar chain by changing the concentration in the range of 0-40mM, or replacing with a glucose, and exchanging the sugar in culture medium for the sugar which exists in sugar chain structures of glycoprotein, such as fructose, a mannose, a galactose, N-acetyl glucosamine, a ribose, fucose, and N-acetyl galactosamine. Or the effectiveness for which it asks by adding while cultivating the polysaccharide containing the sugar which constitutes sugar chains, such as chitosan, dextran sulfate, and an alginic acid, can be acquired. As an approach of changing the sugar chain of glycoprotein, this is a completely new approach.

[0006] It cultivates by choosing the medium composition which was suitable for growth of the cell in the cell which produces target glycoprotein, especially the cell strain which carried out the transformation by transgenics. In order to change the integrated state of a sugar chain, it is desirable to cultivate by sugar concentration lower than the optimal sugar concentration in many cases. Although the culture medium in this case has a desirable serum free medium, it does not interfere, even if it is a blood serum content culture medium. As a culture medium which shows the property excellent in culture of mammalian cells, such as a hybridoma, although MEM, F-hum 10 culture medium, F-hum 12 culture medium, RPMI1640 culture medium, an ERDF culture medium, etc. can be illustrated, the sugar chain of glycoprotein can be changed by making it 0 - 50% concentration of the optimal sugar concentration

used for these culture media. Since the effect by sugar concentration is large, when a blood serum uses an indispensable cell strain for growth, it is desirable [modification of the sugar chain by this invention] to dialyze a blood serum and to remove sugar, so that it may illustrate in the example. When using sugar other than a glucose, the glycoprotein with which the sugar chain was changed can be obtained by transposing to other sugar for [of optimum density / all glucose contents or a part of] growth of a cell. As sugar which shows such effectiveness especially, although monosaccharides, such as fructose, a mannose, a galactose, a glucosamine, and a ribose, can be illustrated, the activity of a galactose, a glucosamine, and a ribose is especially desirable. Moreover, as a polysaccharide, chitosan, dextran sulfate, an alginic acid, heparin, a chitin, a mannan, etc. can be illustrated, and especially chitosan, dextran sulfate, and an alginic acid are desirable. Although an alginic acid may be a salt, especially sodium alginate is desirable.

[0007] If it is the culture approach suitable for a cell, no matter culture of a cell may be what culture approach, it is usable. For example, the suspended cell culture in a tank, the adhesion culture to a micro carrier or an urethane raw material, culture by the hollow fiber, etc. can be illustrated. Moreover, it may cultivate with the culture medium of the conventional glucose concentration suitable for the cell, and you may exchange for the culture medium which changed the class or concentration of sugar into the phase of producing the glycoprotein to which the sugar chain was transformed, very much until a cell becomes a fixed consistency. In the case of a polysaccharide, the solution of a polysaccharide may be added so that it may become 1-1000microg [/ml] concentration into the usual culture medium. The glycoprotein from culture medium can carry out separation purification by the usual purification and the recovery approach. However, since change of a sugar chain is detectable as a difference of the molecular weight of glycoprotein, SDS-gel electrophoresis and the separation purification approaches by the difference of molecular weight, such as the gel filtration approach, are suitable. An example is shown below and this invention is further explained to a detail.

[0008]

[Example 1] This example explains how to change the sugar chain of the antibody which is glycoprotein produced by culture of an antibody production hybridoma. The glycoprotein obtained by especially this example is an antibody from which compatibility differs by the difference in a sugar chain.

(1) The culture medium which made glucose concentration of this culture medium the concentration of 0, 1, 2, 5, 10, 20, and 36mM by making into a basal medium the ERDF culture medium (referring to JP,3-180175,A) which is a serum free medium developed by preparation Murakami and others of a culture medium where sugar concentration differs from sugar composition was prepared. In addition, glucose (Glc) concentration of an ERDF culture medium is made the optimal [20mM(s)] at the time of a cell culture. Moreover, the culture medium which changed the sugar of this ERDF culture medium into fructose (Fru), a mannose (Man), a galactose (Gal), N-acetyl glucosamine (GlcNAc), and a ribose (Rib) was prepared. Furthermore, fetal calf serum (FCS) was dialyzed to phosphate buffered saline (PBS), and FCS (dialysis FCS) which does not contain monosaccharides, such as a glucose, was prepared, and it added so that it might become concentration 5% at each culture medium.

[0009] (2) Homo sapiens-Homo sapiens hybridoma C5TN was used as a production glycoprotein production cell of glycoprotein. C5TN is the substrain of hybridoma HB4C5 (Murakami et al., In Vitro Cell.Develop.Biol., vol.21, 593-596, 1985). Carboxypeptidase (henceforth, CPA), Double stranded DNA (henceforth, DNA), As opposed to Candida citchrome C (henceforth, CytC) It is the cell strain which secretes the Homo sapiens IgM mold antibody which has compatibility (Tachibana, H., et al., Biochem.Biophys.Res.Commun., vol.189, 625-632, 1992). the L chain of the antibody (following C5TN antibody) which this hybridoma secretes -- a lambda chain -- it is -- this invention persons -- CDR1 field of a variable region (VL domain) -Ser-Gly-Asn-Ser-Ser-Asn-Ile-Gly- ** -- it is shown clearly that it has the sugar chain binding site to say. In addition, this cell strain can receive distribution from the Kyushu University agricultural department graduate school cell technology classroom. It is this C5TN in 5ml of each culture medium 5x104 It wound, was crowded with the cell / consistency of ml, and cultivated for 24 hours under 37 degrees C and 5% carbon-dioxide-gas ambient atmosphere, cells were collected, and the culture supernatants cultivated on the still more nearly same conditions for 48 hours were collected.

[0010] (3) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the culture supernatant cultivated by separation each culture medium of the glycoprotein with which sugar chains differ under reduction conditions. Each lambda chain checked by the western blotting which used the ** lambda antibody (drawing 1 , drawing 2). Although four kinds of L chains were detected, they named L1, L2, L3, and L4 from the one where molecular weight is larger, judging from the location of migration. Moreover, only L4 was compounded under the tunicamycin existence which is a sugar chain composition inhibitor. It was presumed that L4 is an antibody which a sugar chain does not combine. Production of the antibody in each culture medium was shown in a table 1.

[0011]

[A table 1]

---------- Culture-medium sugar composition (mM) The type of an L chain ---------- ---------- Glc 0 L1, L3, L4 Glc 1 L1, L3, L4 Glc 2 L1, L3, L4 Glc 5 L1, L2, L3, L4 Glc 10 L1, L2 Glc 20 L1, L2 Glc 36L1, L2 Fru20 L1, L2 Man 20 L1, L2 Gal 20 L1, L2, L4GlcNAc 20L1, L3, L4 Rib 20 L1, L3, L4 ---------- [0012] The glucose concentration in a culture medium or the antibody of L2 which is not obtained by the usual culture condition by change of sugar was able to be obtained. This antibody was separable from other antibodies with SDS-PAGE. This cell produces the antibody containing a lambda chain (L chain) with four kinds of molecular weight, as mentioned above, and the difference of this lambda chain (L chain) molecular weight is based on the molecular weight difference of a sugar chain.

[0013]

[Example 2]

The sugar chain had combined with the L chain and the property change C5TN antibody of the antibody by the difference in a sugar chain was able to be used as the sugar chain from which molecular weight as shown in (3) differs this sugar chain by this invention. It checked as follows that antibody specificity changed with modification of this sugar chain. The antigenic specificity of an antibody can measure an antigen with the enzyme immunoassay (ELISA) using the microplate which carried out the coat (H. Murakami et al., Animal Cell Technology:Basic & Applied Aspects, 547- 551, 1992, Kluwer Acdemic Publishers). The solution with a C4TN antibody of 200 ng/ml cultivated by each culture medium was prepared, and the antigenic specificity of this antibody solution was measured. Measured value is Glc. The reactivity of the antibody cultivated and obtained by 20mM was expressed with the percentage made into 100%. The result was shown in drawing 3 and drawing 4 . Although CPA compatibility and Cytc compatibility fell with the increment in L3 and L4 when changing the content of Glc, DNA compatibility reached the peak in 2mM(s). Moreover, when the sugar composition in a culture medium was changed, the compatibility of an antibody showed a completely different result from the antibody cultivated and obtained by Glc by Gal, GlcNAc, and Rib addition (drawing 3 , drawing 4). Since the structure of the sugar chain combined with an antibody changed, this was presumed.

[0014]

[Example 3] The hybridoma C5TN cell was cultivated on the same conditions as an example 1 using the ERDF basic serum free medium which adjusted glucose concentration to 10mM(s). That is, it is hybridoma C5TN 5x104 It wound, and was crowded with the cell / consistency of ml, and culture was performed for 6 hours. After having collected cells after that, moving to chitosan addition (1microg/(ml)), dextran sulfate addition (100microg/(ml)), or alginic-acid addition (100microg/(ml)) culture medium (ERDF) and performing culture for further 48 hours, culture supernatants were collected and dialysis concentration of this was carried out. A culture condition is 37 degrees C and 5%CO2. It carried out like the example 1 under the ambient atmosphere. Moreover, contrast cultivated only by ERDF. SDS-PAGE performed electrophoresis for this concentration liquid on reduction conditions, and the L chain of an antibody was specifically detected by western blotting by the anti-Homo sapiens lambda L chain antibody. The result was shown in drawing 5 . By adding each polysaccharide, the L chain of 28KD(s) and 30KD(s) increased as compared with culture of only a glucose. This was the result of proving that the sugar chain of an antibody is changing. Moreover, each obtained antibody was only an antibody of an IgM mold.

[0015]
[Example 4] Change of the antigen compatibility of the antibody from which the sugar chain produced in the example 3 changed was checked by the approach indicated in the example 2. It measured similarly using the microplate which carried out the coat of the antigen, and the reactivity over each antigen was measured by the ELISA method. The reactivity over each antigen observed change of coloring by the ELISA method by 405nm absorbance change. The result was shown in drawing 6 -8. As for the antibody, it was checked by change of a sugar chain that reactivity with an antigen is changing.
[0016]
[Effect of the Invention] In case an animal cell is cultivated and glycoprotein is produced by this invention, by controlling culture conditions, such as a presentation of monosaccharides, such as a ribose in a culture medium, a galactose, and a glucosamine, or sugar concentration, the class or molecular weight of a sugar chain added to protein can be changed, and the glycoprotein from which the property changed can be produced. Moreover, an alginic acid, dextran sulfate, and a polysaccharide like chitosan can be added in a culture medium, the class of sugar chain or molecular weight can be changed, and the glycoprotein from which the property changed can be produced. The glycoprotein from which the class of sugar chain or molecular weight differs by this approach can be obtained. Moreover, the bioactive of glycoprotein can also be changed by the alteration of a sugar chain.

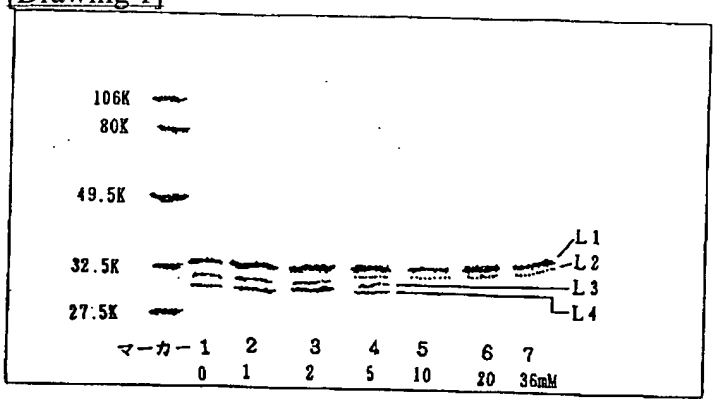
[Translation done.]

DESCRIPTION OF DRAWINGS

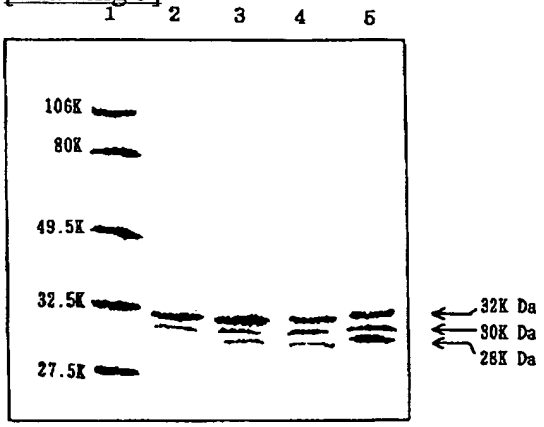
[Brief Description of the Drawings]
[Drawing 1] The detection pattern by the SDS-PAGE western blotting of lambda mold antibody obtained from the C5TN cell which changed and cultivated glucose concentration according to the example 1 is shown.
[Drawing 2] The detection pattern by the SDS-PAGE western blotting of lambda mold antibody obtained from the C5TN cell which changed the glucose into other sugar and cultivated it according to the example 1 is shown.
[Drawing 3] Change of the antigen compatibility of the antibody obtained from the C5TN cell which changed and cultivated glucose concentration according to the example 2 is shown.
[Drawing 4] The antigen compatibility of the antibody obtained from the C5TN cell which changed the glucose into other sugar and cultivated it according to the example 2 is shown.
[Drawing 5] The detection pattern by the SDS-PAGE western blotting of the Homo sapiens lambda mold antibody obtained from the example 3 is shown.
[Description of Notations]
Lane 1: Molecule size marker
Lane 2: Contrast
Lane 3: Chitosan addition (1microg/(ml))
Lane 4: Dextran sulfate addition (100 mug/ml)
Lane 5: Alginic-acid addition (100 mug/ml)
[Drawing 6] Change of the compatibility over CPA of the antibody obtained according to the example 3 is shown.
[Drawing 7] Change of the compatibility over CytC of the antibody obtained according to the example 3 is shown.
[Drawing 8] Change of the compatibility over dsDNA of the antibody obtained according to the example 3 is shown.
[Description of Notations]
- O- Contrast
- **- Chitosan addition (1microg/(ml))
- -- Dextran sulfate addition (100 mug/ml)
- **- Alginic-acid addition (100 mug/ml)

[Translation done.]

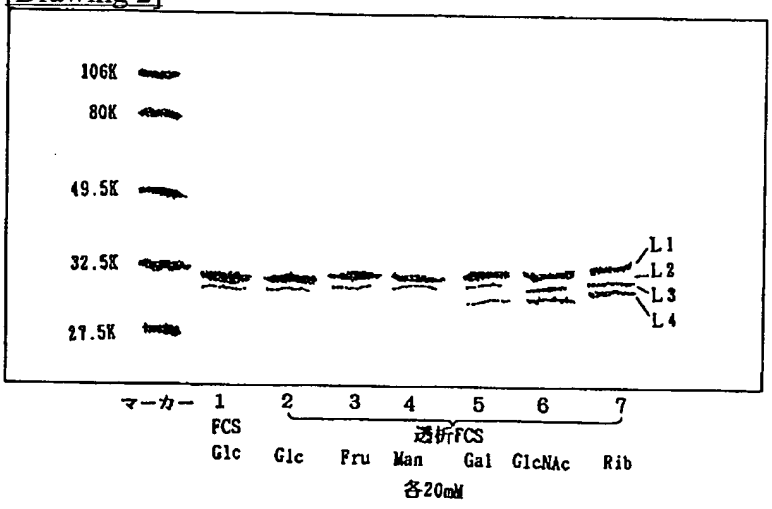
[Drawing 1]



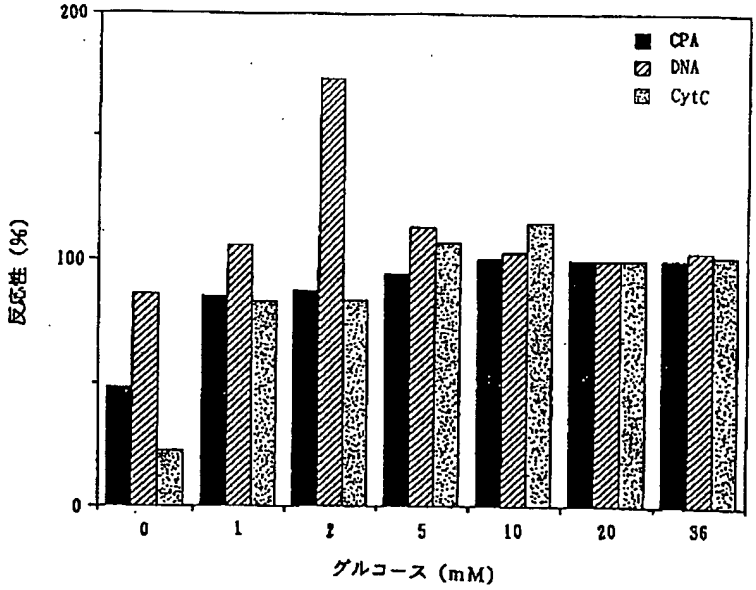
[Drawing 5]



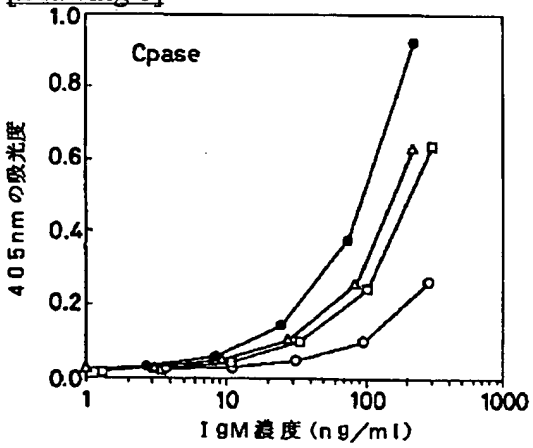
[Drawing 2]



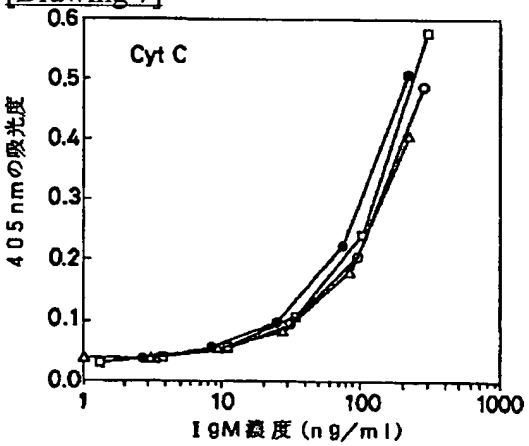
[Drawing 3]



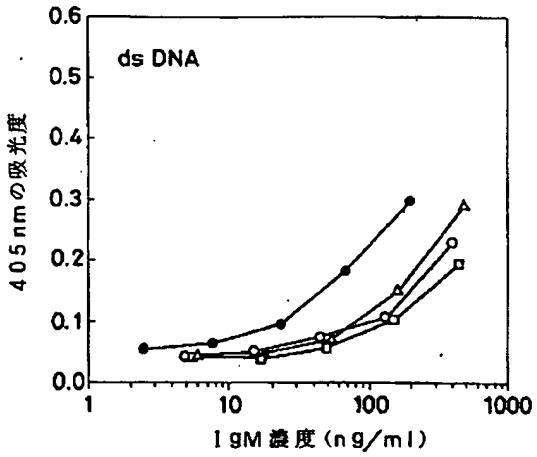
[Drawing 6]



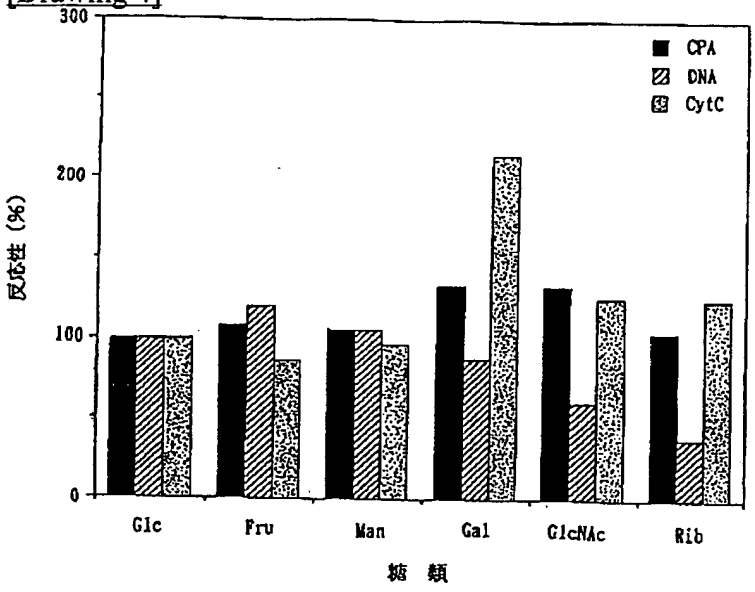
[Drawing 7]



[Drawing 8]



[Drawing 4]



[Translation done.]

* NOTICES *

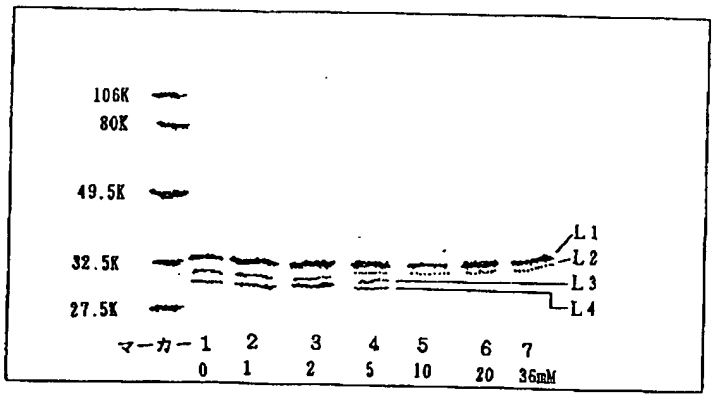
1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

CLAIMS

[Claim(s)]
[Claim 1] The process of the glycoprotein characterized by changing the class or molecular weight of a sugar chain combined with protein by changing the sugar composition and/or sugar concentration in a culture medium in cultivating an animal cell in a culture medium and producing glycoprotein.

[Translation done.]

Drawing selection drawing 1



[Translation done.]

English abstract of Cited Document 1

PatentWeb Home | Edit Search | Return to Patent List | Help | Include in patent order

MicroPatent(R) Worldwide PatSearch: Record 1 of 1

[no drawing available]

Family Lookup

JP08252592
PRODUCTION OF GLYCOPROTEIN
SNOW BRAND MILK PROD CO LTD
Inventor(s): TACHIBANA HIROFUMI ;MURAKAMI HIRONORI ;NIIMOTO YOJI ;DOSEMARI SHUNICHI
Application No. 06031019 , Filed 19940202 , Published 19941021

Abstract:

PURPOSE: To alter the kind and molecular weight of bounded sugar chain by changing the sugar composition and/or sugar concentration in a medium for the culture of animal cell to produce a glycoprotein.

CONSTITUTION: The composition and/or concentration of a sugar such as ribose, galactose and glucosamine in a medium are changed in the culture of an animal cell in the medium to produce a glycoprotein. The kind or the molecular weight of the sugar chain of the glycoprotein can be altered by this process to obtain glycoproteins having different activity or stability.

Int'l Class: C12P02100 C12P02108 C12P02100 C12R00191 C12P02108 C12R00191

Priority: JP 05 44587 19930209

MicroPatent Reference Number: 002225034
COPYRIGHT: (C) 1994 JPO

PatentWeb Home | Edit Search | Return to Patent List | Help

For further information, please contact:
Technical Support | Billing | Sales | General Information